Kinetics of Cellulose Digestion by *Fibrobacter Succinogenes* S85

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Introduction

Simple-stomached animals digest cellulose poorly, but ruminants, by exploiting cellulolytic microorganisms, have developed a much greater capacity for cellulose digestion. The relationship between ruminants and ruminal bacteria is clearly symbiotic. The animal provides the bacteria with a habitat for growth, the rumen, and the bacteria supply the animal with volatile fatty acids and microbial protein. The enzymology of cellulases has been confounded by variations in cellulose structure and the methodology used to measure cellulose hydrolysis. Early work was based on gravimetric procedures, but these methods are inherently insensitive. Reducing-sugar release has been used, but linear rates could only be obtained if the extent of cellulose digestion was less than 4%. Many "cellulase" assays used carboxylmethylcellulose (CMC), a soluble derivative, but many CMCases have little if any activity on crystalline cellulose. Both surface area and crystallinity can affect the rate of cellulose digestion, but Weimer concluded that surface area was a much more important parameter for ruminal cellulose digestion than was crystallinity. Surface area is sometimes estimated from particle size, but these estimates can be biased by the shape of the particle. Nitrogen absorption and solute exclusion are in theory more accurate, but both of these methods can be problematic. The following experiments were performed with *Fibrobacter succinogenes* S85, one of the most active cellulolytic bacteria isolated from the rumen. The experiments were designed to: 1) define conditions necessary to estimate rates of cellulose digestion, 2) determine the impact of cellulose surface area on rates of cellulose digestion, and 3) evaluate the relationship between culture status (growing versus stationary) and the rate of cellulose digestion.

Materials and Methods

F. succinogenes S85 was grown anaerobically in a defined medium. Exponentially growing cells were harvested by centrifugation, washed and resuspended in fresh media containing different concentrations of cellulose (from 1 to 10 mg/ml). After incubation for 60 min at 39°C, the suspensions were centrifuged. Succinate in the cell-free supernatant was measured by HPLC. Three commercial pure celluloses were used. Sigmacell 100 (Sigma Chemical Co.), Whatman no. 1 filter paper and Avicel type PH101 (FMC Co., Philadelphia). In addition two of them were pre-treated as follow: Whatman no. 1 filter paper (20 mg/ml) was ball-milled for 9 days using a 800 ml Mill Jar, "roller-type" (Norton, Chemical Process Products Division, Ohio) and microcrystalline cellulose, Avicel type PH 101, was hydrolyzed with hydrochloric acid and regenerated. Cellulose suspensions (10 mg/ml) were mixed with 2% Congo red solution to final concentrations ranging from 0.005 to 6.0 mg/ml of Congo red. The cellulose suspensions were centrifuged and the dye in the supernatant was monitored at 500 nm. The extinction coefficient of Congo red was 23,300 M^{-1} cm^{-1}. Exponentially growing cells of *F. succinogenes* S85 were centrifuged, washed and resuspended in fresh media containing 0.2 mg/ml of 4-thiocellobiose (Sigma Chemical Co.). Cellulose was added, the cells were incubated for 60 min at 39°C and cellulose-dependent succinate production was detected as described above. Cells were treated with 0.2 N NaOH (100°C, 10 min) and protein was determined by the method of Lowry et al.

Results

Growing cultures of *F. succinogenes* S85 digested cellulose at a rapid rate, but non-growing cells and cell extracts did not have detectable crystalline cellulase activity. Cells that had been growing exponentially on cellobiose initiated cellulose digestion and succinate production immediately, and cellulose-dependent succinate production could be used as an index of enzyme activity against crystalline cellulose. Cells incubated with cellulose never produced cellobiose, and cells that were pre-incubated for a short time with thiocellobiose lost their ability to digest cellulose (competitive inhibition, K_i of only 0.2 mg/ml or 0.56 mM). Based on these results, the crystalline cellulases

of *F. succinogenes* were very sensitive to feedback inhibition. Different cellulose sources bound different amounts of Congo red, and the binding capacity was HCl-regenerated cellulose > ball-milled cellulose > Sigmacel > Avicel > filter paper. Congo red binding capacity was highly correlated with the Vmax values of cellulose digestion and inversely related to Km. Congo red (250 mg/ml) did not inhibit the growth of *F. succinogenes* S85 on cellobiose, but this concentration of Congo red inhibited the rate of ball-milled cellulose digestion. A Lineweaver Burk plot of ball-milled cellulose digestion rate versus the amount of cellulose indicated that Congo red was a competitive inhibitor of cellulose digestion (K_i was 250 mg/ml).

Discussion

F. succinogenes S85 is one of the most active cellulolytic bacteria ever isolated from the rumen, but cell extracts have little or no crystalline cellulase activity. A variety of b-glucanases have been either isolated or cloned from *F. succinogenes* S85, but none of these enzymes had crystalline cellulase activity per se. Preliminary experiments indicated that cell extracts *of F. succinogenes* S85 were unable to produce reducing sugar from ball milled cellulose. Since cells limited by ammonia or branched chain volatile fatty acids also lost their ability to digest cellulose as soon as growth ceased, it appeared that only actively growing cells had crystalline cellulase activity.

When *F. succinogenes* S85 cultures that had been growing exponentially on cellobiose were harvested by centrifugation and re-suspended in medium containing cellulose, it was possible to measure the initial rate of succinate production. Experiments with ball-milled cellulose indicated that the cellulose-dependent succinate production rate was comparable to the succinate production of cultures growing on cellulose (53 versus 60 nmol succinate/mg protein/min). The rate of cellulose-dependent succinate production was linear so long as the incubation time was less than 60 min and the cell concentration was less than 150 mg protein/ml.

The cellulase activity of *F. succinogenes* S85 was directly linked to cellobiose metabolism. Little decline in "cellulase activity" was noted until the rate of cellobiose fermentation was less than 50 nmol succinate per mg protein per min, but there was a linear and proportional decrease in cellulose degradation at slower rates of cellobiose metabolism. The cellulases of non-ruminal microorganisms can be inhibited by cellobiose, but large amounts are needed to cause significant inhibition. The idea that *F. succinogenes* cellulases might be unusually sensitive to feedback inhibition was supported by the effect of thiocellobiose, a non-metabolizable cellobiose analog. Exponentially growing cells that were treated with thiocellobiose lost their ability to degrade cellulose, and there was no detectable increase in cellobiose. Thiocellobiose was a competitive inhibitor of cellulose degradation, and the K_i was only 0.2 mg/ml or 0.56 mM.

The V_{max} of cellulose digestion varied 8-fold when different cellulose sources were provided, and this rate was highly correlated with the ability of the cellulose to bind Congo red. Congo red is a polyphenolic, sulfonated azo-dye that has been used as an pH indicator, but it also binds to the surface of insoluble plant carbohydrates. Based on these results, it appeared that cellulose surface area was the primary factor affecting cellulase activity. Cellulose sources with high V_{max} values tended to have low K_m values and vice versa, and this result is not surprising. If the turnover rate of cellulose is rapid (high V_{max}), it would take less cellulose to saturate the cellulase (low K_m). Avicel is frequently used as a substrate for crystalline cellulase assays, but a plot of Congo red binding versus K_m indicated that Avicel was abnormal. Avicel is a microcrystalline cellulose, but the crystalline cellulase of *F. succinogenes* S85 is cell associated that cannot penetrate into small pores.

Conclusion

Crystallinity seems to have little impact on ruminal fiber digestion rate, but surface area can have a major impact.